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Struvite crystal growth inhibition by trisodium citrate and the formation of chemical complexes in growth solution

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ABSTRACT

Effect of trisodium citrate on the crystallization of struvite was studied. To evaluate such an effect an experiment of struvite growth from artificial urine was performed. The investigations are related to infectious urinary stones formation. The crystallization process was induced by the addition of aqueous ammonia solution to mimic the bacterial activity. The spectrophotometric results demonstrate that trisodium citrate increases induction time with respect to struvite formation and decreases the growth efficiency of struvite. The inhibitory effect of trisodium citrate on the nucleation and growth of struvite is explained in base of chemical speciation analysis. Such an analysis demonstrates that the inhibitory effect is related with the fact that trisodium citrate binds NH_4^+ and Mg^{2+} ions in the range of pH from 7 to 9.5 characteristic for struvite precipitation. The most important is the $MgCit^-$ complex whose concentration strongly depends on an increase in pH rather than on an increase in citrate concentrations.

1. Introduction

Struvite (magnesium ammonium phosphate hexahydrate; $MgNH_4PO_4 \cdot 6H_2O$) is an inorganic material and its crystallization is more and more widely investigated for several reasons. The major and the most important reason for which struvite is widely investigated is the fact that it is the main component of so-called infectious urinary stones [1]. Struvite is formed when the urinary tract becomes colonized by bacteria (e.g. *Proteus*) which produce an enzyme called urease. Urease catalyzes the hydrolysis of urea, $(NH_2)_2CO$ to form ammonia, NH_3 [2]. As a consequence, the pH of the urine rises. The increasing urinary pH leads to an elevation of the concentration of the NH_4^+ and PO_4^{3-} ions. These ions together with the ions of magnesium Mg^{2+} present in the urine lead to the crystallization of struvite according to the reaction:

$$Mg^{2+} + NH_4^+ + PO_4^{3-} + 6H_2O \overset{pH \geq 7.2}{\Longrightarrow} MgNH_4PO_4 \cdot 6H_2O. \quad (1)$$

Struvite formation is usually associated with the precipitation of carbonate apatite ($Ca_{10}(PO_4)_6CO_3$; CA), because the ions of calcium Ca^{2+} are present in the urine and CO_3^{2-} ions occur as a result of pH elevation.

Struvite together with small amount of CA (up to 10%) forms the so-called infectious or struvite stones which are related to a urinary tract infection. This kind of stones constitutes up to 20% of all urinary stones [3]. Struvite stones may grow rapidly and, if not adequately treated, can develop into a large stone that fills the entire intra-renal collecting system. Patients with infectious stones who receive no treatment have about a 50% chance of losing a kidney [4,5]. The infectious urinary stones represent serious health problem, and more often are a greater danger than metabolic stones because of renal tissue damage associated with infectious urinary stones and the recurrence after treatment on the level of 50% [3].

Therefore, in recent years, many studies on the crystallization of struvite precipitation have been undertaken. Different substances were analyzed to show their influences on these processes and provide effective treatment methods. For example, the inhibition effect of phosphocitrate and acetohydroxamic acid on struvite precipitation was shown [6,7]. Also, several phytotherapeutic compounds have recently been investigated to treat or prevent bacterial infections which may lead to urinary stones. For example, the growth inhibition studies of struvite in the presence of the juice of *Citrus medica* Linn. [8] and in the presence of the herbal extracts of *Commiphora wightii* [9], *Rotula aquatica* Lour. [10], *Boerhaavia diffusa* Linn. [11] and curcumin [12] are successfully carried out.

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In this paper we focus on the trisodium citrate ($Na_3C_6H_5O_7$), the sodium salt of citric acid, and its effects on nucleation and growth of struvite. Trisodium citrate abbreviated as citrate is a normal component of urine of healthy person. Typical daily excretion ranges from 0.5 mM to 5 mM [13]. There were many studies for the citrate inhibitory effect against calcium oxalate i.e. the compound which occurs in metabolic urinary stones as a result of metabolic imbalance [14]. These studies have shown that citrate has ability to inhibit the nucleation and crystallization processes of this kind of compound. Citrate was also examined in relation to struvite crystallization [15–17]. The study [15] demonstrates that citrate has an inhibitory effect on the growth of struvite. The inhibitory effect in this study was estimated based on the struvite morphology and habit observation. It is known that high growth rates induce the appearance of dendritic or X-shaped crystals. When growth rates decrease, the more balanced three-dimensional single crystals appear. Such a transition from dendritic to single crystals was interpreted as an evidence of inhibitory action by citrate [15]. An inhibitory effect of citrate was also studied using Coulter counter techniques and optical microscopy [16]. The studied citrate concentrations were equal to 1, 2, 3 and 4 mM [16]. The results demonstrate that citrate delays the nucleation process, but the increase in particle number is less pronounced [16]. The effect of citrate with concentrations in the range 0.6–11.4 mM was also studied in relation to struvite crystallization [17]. The obtained results demonstrate that citrate does not influence the crystallization process but it influences pH.

The above presented review of literature shows that the effect of citrate on struvite crystallization needs further evaluation. Therefore, in the present study we describe the effect of citrate on the crystallization of struvite using the objective spectrophotometric methods associated with optical microscopy observations. The present study links experimental crystal growth data with theoretical speciation analysis of chemical complexes which may be formed in the case when crystals occur in artificial urine in the presence of different concentrations of citrate. Such a speciation analysis of chemical complexes has not been done by now but it is very important because it provides insights into why citrate is an inhibitor of struvite crystals.

2. Materials and methods

The solution of artificial urine usually used for experiments is composed from the following components, with concentrations (g/l) in brackets: $CaCl_2 \cdot 2H_2O$ (0.651), $MgCl_2 \cdot 6H_2O$ (0.651), NaCl (4.6), Na_2SO_4 (2.3), KH_2PO_4 (2.8), KCl (1.6), NH_4Cl (1.0), $Na_3C_6H_5O_7$ (0.65), $C_2Na_2O_4$ (0.023), urea (25.0), creatine (1.1) and tryptic soy broth (10.0). The tryptic soy broth (TSB) is used to stimulate the bacterial growth. The present study was done without bacteria, but TSB was added to the solution of artificial urine for simplification of future comparative study in the case of research in the presence of bacteria. TSB does not have an effect on the crystallization processes. Such a composition of artificial urine is widely accepted in literature [18,19]. The composition of artificial urine used for the experiments presented in this paper is modified. The modification consists in this that the calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$) was not added. This is related with the fact that in the present study we focus on struvite crystallization only. The presence of calcium chloride dihydrate in the solution of artificial urine causes the carbonate apatite precipitation also as described in Section 1. Carbonate apatite precipitates earlier (for lower pH) than struvite and its precipitation disturbs the spectrophotometric measurements. For this reason calcium chloride dihydrate was not added in the present study.

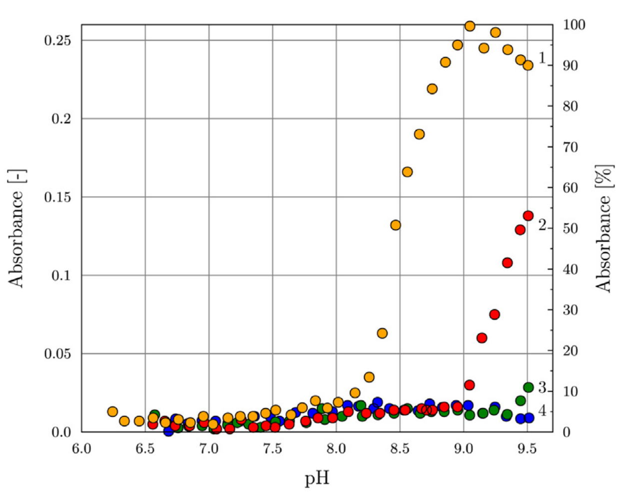

Fig. 1. Absorbance of artificial urine versus pH for baseline (2.52 mM citrate concentration) – symbol 1 and for elevated citrate concentrations: 20 mM, 40 mM and 60 mM – symbols 2, 3 and 4, respectively.

The artificial urine was prepared by dissolving chemicals (Sigma Aldrich) of reagent-grade purity in redistilled water. Then, the solution was filtered through a membrane filter with pore size of 0.2 μm. The solution of artificial urine was stored for a maximum 48 h at 4 °C. The initial pH of artificial urine was adjusted to a value of 5.8. The crystallization process occurs after consecutive addition of aqueous ammonia solution (1.2 M) to mimic urease activity. Such an addition causes an increase in pH value, as it occurs in the case of real urinary tract infection. The experiment is made for pH value equal to 9.5. This is the highest value of pH which may be reached in the case of the experiment in the presence of bacteria. In the case of the presence of bacteria such a value of pH is achieved after 24 h of experiment and it does not increase further [20]. The pH of the solution of artificial urine was screened along the experiments using digital pH-meter (Elmetron CPC-401). Using this equipment the pH measurements are done with accuracy of 0.01. In the paper, the average results with accuracy of 0.1 are given. The experiments were conducted under thermostated conditions at 37 ± 0.5 °C. The temperature was kept constant by circulating water from a constant temperature water bath. The experiments were done at least in triplicate for the assessment of repeatability.

The concentration of citrate in natural human urine ranges from 0.5 mM to 5 mM [6], while in artificial urine of composition presented above it is equal to 2.52 mM. This concentration 2.52 mM is considered as baseline in the present study. The elevated citrate concentration tested was equal to 20, 40 and 60 mM. The calculated amounts of citrate were dissolved in artificial urine and made up to volumes of 25 ml in volumetric flasks. In order to estimate the effect of elevated concentration of citrate on nucleation and growth of struvite, the turbidity of artificial urine with normal and elevated concentration of citrate was measured as the absorbance of light of defined wavelength. The optimized wavelength was estimated to be equal to 530 nm. In our experiments the absorbance was measured with spectrophotometer Spekol 11 (Carl Zeiss) using glass cuvettes with path length of 10 mm and is given in absorbance units. During all the growth experiments the samples were taken at regular intervals and observed by optical microscopy Opta Tech MN 800.

A speciation analysis of complexes in artificial urine for the normal (2.52 mM) and elevated concentrations (20, 40 and 60 mM) of citrate was carried out by using computer code Hyperquad Simulation and Speciation (HySS) [21]. Using stability constant complex forming in the artificial urine and the initial molar concentrations of the ions constituting the composition of the complexes, the program makes it possible to calculate molar